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Morphology and phylogeny of *Pseudocercospora kamalii* sp. nov., a foliar pathogen on *Terminalia* from India

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Abstract — *Pseudocercospora kamalii* (*Mycosphaerellaceae, Capnodiales*) is associated with severe leaf spot disease of *Terminalia chebula* (*Combretaceae*) in natural forests of Mahabaleshwar in the Western Ghats of India. Morphological characterization reveals that the new species proposed has some affinity with the species of genus *Prathigada*. However, based on the molecular sequence data (LSU and ITS 1-5.8S-ITS 2) generated in the present study, we established its identity as a species of *Pseudocercospora*. The species is also compared morphologically with other *Pseudocercospora* species reported on *Terminalia* from different parts of the world.

Key words — anamorphic fungi, *Mycosphaerella*, plant pathogen

Introduction

The microfungal diversity of northern Western Ghats has been well explored in the recent past (Karandikar & Singh 2010, Rajeshkumar et al. 2010, 2011; Singh et al. 2009, 2010; Waingankar et al. 2008). During January 2011 a survey was conducted to explore the microfungal diversity in the natural forests of Mahabaleshwar, situated in the northern part of the Western Ghats, India, at 17°58′N 73°43′E. An unusual synnematous cercosporoid species was discovered that caused a severe foliar disease in *Terminalia chebula*. The present study aimed to identify the causal agent of the foliar infection on *Terminalia* in northern Western Ghats, India using morphological and molecular methods.

Materials & methods

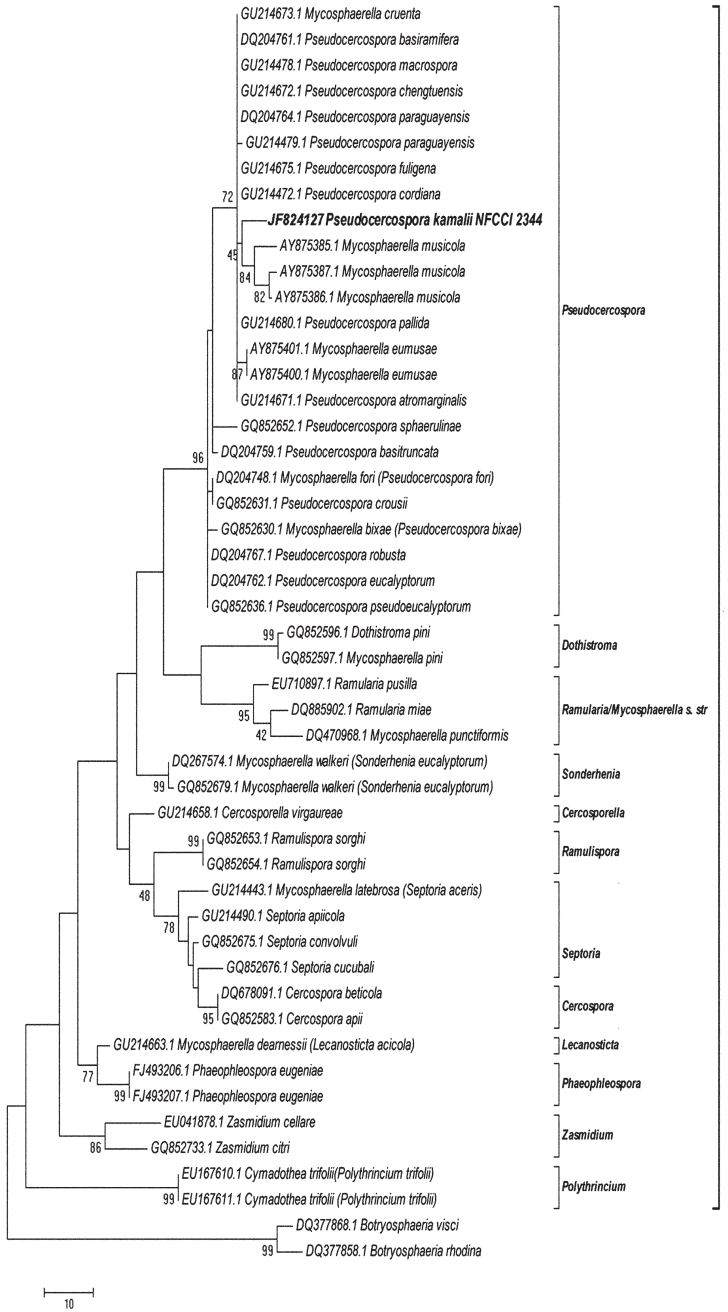
Isolates and morphology— Synnemata of the fungus were directly isolated from the surface of fallen fruits and observed under a Nikon Binocular stereo microscope (Model SMZ-1500 with Digi-CAM, Japan). Single conidial cultures were established

on 2% potato dextrose agar plates (PDA; Crous et al. 2009a). For morphotaxonomic studies and photomicrographs an Olympus (Model CX-41, Japan) microscope was used. Conidia and conidiomata were mounted in lactic acid cotton blue and measured using an ocular micrometer, with 30 observations per structure. Colony characteristics in culture were studied on two different media: 2% malt extract agar (MEA) and PDA (Crous et al. 2009a). Herbarium specimens were deposited in the Ajrekar Mycological Herbarium (AMH); the culture was accessioned and preserved in the National Fungal Culture Collection of India (NFCCI; WDCM-932), Agharkar Research Institute, Pune, India under accession number NFCCI 2344.

Polymerase chain reaction (pcr) and sequencing— Total DNA was extracted from cultures grown on PDA plates for two weeks at 25 ºC, using a FastDNA® SPIN kit as per the manufacturer's instructions (MP Biomedicals GmbH, Germany). Fragments containing the region encoding the 28S nrDNA (LSU) and ITS 1-5.8S nrDNA-ITS 2 (ITS) were amplified using primer pairs LROR (Rehner & Samuels 1994) and LR7 (Vilgalys & Hester 1990) for LSU; ITS4 and ITS5 (White et al. 1990) for ITS. DNA amplification was performed in a 25 µl reaction using 2 µl of template DNA (10–25 ng), 0.5 U of Taq DNA polymerase (Genei, Bangalore, India), 2.5 µl of 10× Taq DNA polymerase buffer, 0.5 µl of 200 µM of each dNTPs (Genei, Bangalore, India), 0.5 µl of 10 pmol primer, H_2O (Sterile Ultra Pure Water, Sigma) to make up 25 µl. Amplification in an Eppendorf Mastercycler AG used the following parameters: 5 min at 95 °C; 30 cycles of 1 min at 95 °C, 30s at 56 °C, and 1 min at 72 °C for the ITS region amplification; and final 7-min extension step at 72 °C. DNA amplification of LSU followed the ITS conditions except for a 52 °C annealing temperature. The PCR products were purified with an Axygen PCR cleanup kit (Axygen Scientific Inc, CA, USA) and sequenced with the same primers using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The sequencing reactions were run on an ABI 3100 automated DNA sequencer (Applied Biosystems, USA).

Sequence alignment and phylogenetic analysis— LSU & ITS sequences from *P. kamalii* were aligned manually using the text editor option of the Molecular Evolutionary Genetics Analysis (MEGA) software v4.0. (Tamura et al. 2007). The manually edited NFCCI 2344 sequences were deposited in the NCBI sequence nucleotide database (ITS: JF 824126, LSU: JF 824127). They were also subjected to a BLAST search of the NCBI Genbank nucleotide database. The ITS sequences were aligned using Clustal W together with the homologous regions of ITS of closely related species of *Pseudocercospora* Speg. and *Mycosphaerella* s.l. For ITS, the matrix was analyzed with the Maximum Parsimony

Fig. 1. Phylogenetic tree based on aligned LSU sequences of *Pseudocercospora* and its teleomorph, *Mycosphaerella* s.l. The consistency index (0.566343), the retention index (0.821571), and the composite index (0.479675) were calculated in MEGA for all sites and parsimony-informative sites (in parentheses). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The MP tree was obtained using the Close-Neighbor-Interchange algorithm with search level 3 in which the initial trees were obtained with the random addition of sequences (10 replicates). The tree is drawn to scale, with branch lengths calculated using the average pathway method and are in the units of the number of changes over the whole sequence. All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option).


GU214673.1 Mycosphaerella cruenta
DQ204761.1 Pseudocercospora basiramifera
GU214478.1 Pseudocercospora macrospora
GU214672.1 Pseudocercospora chengtuensis
DQ204764.1 Pseudocercospora paraguayensis
GU214479.1 Pseudocercospora paraguayensis
GU214675.1 Pseudocercospora fuligena
GU214472.1 Pseudocercospora cordiana
JF824127 Pseudocercospora kamalii NFCCI 2344
AY875385.1 Mycosphaerella musicola
AY875387.1 Mycosphaerella musicola
AY875386.1 Mycosphaerella musicola
GU214680.1 Pseudocercospora pallida
AY875401.1 Mycosphaerella eumusae
AY875400.1 Mycosphaerella eumusae
GU214671.1 Pseudocercospora atromarginalis
GQ852652.1 Pseudocercospora sphaerulinae
DQ204759.1 Pseudocercospora basitruncata
DQ204748.1 Mycosphaerella fori (Pseudocercospora fori)
GQ852631.1 Pseudocercospora crousii
GQ852630.1 Mycosphaerella bixae (Pseudocercospora bixae)
DQ204767.1 Pseudocercospora robusta
DQ204762.1 Pseudocercospora eucalyptorum
GQ852636.1 Pseudocercospora pseudoeucalyptorum
GQ852596.1 Dothistroma pini
GQ852597.1 Mycosphaerella pini
EU710897.1 Ramularia pusilla
DQ885902.1 Ramularia miae
DQ470968.1 Mycosphaerella punctiformis
DQ267574.1 Mycosphaerella walkeri (Sonderhenia eucalyptorum)
GQ852679.1 Mycosphaerella walkeri (Sonderhenia eucalyptorum)
GU214658.1 Cercosporella virgaureae
GQ852653.1 Ramulispora sorghi
GQ852654.1 Ramulispora sorghi
GU214443.1 Mycosphaerella latebrosa (Septoria aceris)
GU214490.1 Septoria apiicola
GQ852675.1 Septoria convolvuli
GQ852676.1 Septoria cucubali
DQ678091.1 Cercospora beticola
GQ852583.1 Cercospora apii
GU214663.1 Mycosphaerella dearnessii (Lecanosticta acicola)
FJ493206.1 Phaeophleospora eugeniae
FJ493207.1 Phaeophleospora eugeniae
EU041878.1 Zasmidium cellare
GQ852733.1 Zasmidium citri
EU167610.1 Cymadothea trifolii(Polythrincium trifolii)
EU167611.1 Cymadothea trifolii (Polythrincium trifolii)
DQ377868.1 Botryosphaeria visci
DQ377858.1 Botryosphaeria rhodina
Pseudocercospora
Dothistroma
Ramularia/Mycosphaerella s. str
Sonderhenia
Cercosporella
Ramulispora
Septoria
Cercospora
Lecanosticta
Phaeophleospora
Zasmidium
Polythrincium
Mycosphaerellaceae
10

method using the Tamura model (Tamura et al. 2007) to calculate the sequence divergence, and the bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option in MEGA4).

Results

DNA phylogeny

The alignment for the *Pseudocercospora* LSU phylogenetic analyses comprised a total of 481 positions in the final dataset, of which 128 were parsimony informative. The LSU sequence analysis presented here (Fig. 1) reveals the systematic position of the new species within *Pseudocercospora,* along with its 11 closely allied sister genera in *Mycosphaerellaceae.* The LSU phylogenetic sequence analysis implied 12 main clades: *Pseudocercospora*, *Dothistroma* Hulbary, *Ramularia* Unger/*Mycosphaerella* s. str., *Sonderhenia* H.J. Swart & J. Walker, *Cercosporella* Sacc., *Ramulispora* Miura, *Septoria* Sacc., *Cercospora* Fresen., *Lecanosticta* Syd., *Phaeophleospora* Rangel, *Zasmidium* Fr., and *Polythrincium* Kunze. All *Pseudocercospora* species clustered together to form a major clade. The new species, *P. kamalii,* clustered close to *Mycosphaerella musicola* R. Leach ex J.L. Mulder in the major *Pseudocercospora* clade. *Botryosphaeria visci* (Kalchbr.) Arx & E. Müll. (DQ 377868.1) and *B. rhodina* (Berk. & M.A. Curtis) Arx (DQ 377858.1) in the *Botryosphaeriaceae* served as outgroup taxa.

For the phylogenetic analysis of closely related *Pseudocercospora* species, the ITS sequence data alignment of 487 bp included 452 positions in the final data set. The analysis of ITS sequence shown in Fig. 2 reveals a significant association of *Pseudocercospora* representatives, some with *Mycosphaerella*-like teleomorphs. The ITS sequence analysis implied four major clades, with *Pseudocercospora eumusae* Crous & Mour. (= *Mycosphaerella eumusae* Crous & Mour.) forming a unique clade (94% bootstrap support). However, *P. tereticornis* Crous & Carnegie, *P. cruenta* (Sacc.) Deighton, *P. pallida* (Ellis & Everh.) H.D. Shin & U. Braun, *P. casuarinae* Crous & R.G. Shivas, *P. elaeodendri* (G.P. Agarwal & Hasija) Deighton, *P. fuligena* (Roldan) Deighton, *P. chengtuensis* (F.L. Tai) Deighton, and *P. atromarginalis* (G.F. Atk.) Deighton clustered together to form a major group in the *Mycosphaerellaceae*. *Pseudocercospora musae* (Zimm.) Deighton (= *Mycosphaerella musicola*) also formed a unique clade (100% bootstrap support). Our new species, *P. kamalii,* (66% bootstrap support), separated as sister to the *Pseudocercospora musae* (AY 646475.1 & AY 646474.1) clade. *Cladosporium herbarum* (Pers.) Link (= *Davidiella tassiana* (De Not.) Crous & U. Braun) (DQ 289799.2) was chosen as the outgroup, as it belongs to the family *Davidiellaceae* that is allied to the *Mycosphaerellaceae* (Crous et al. 2006).

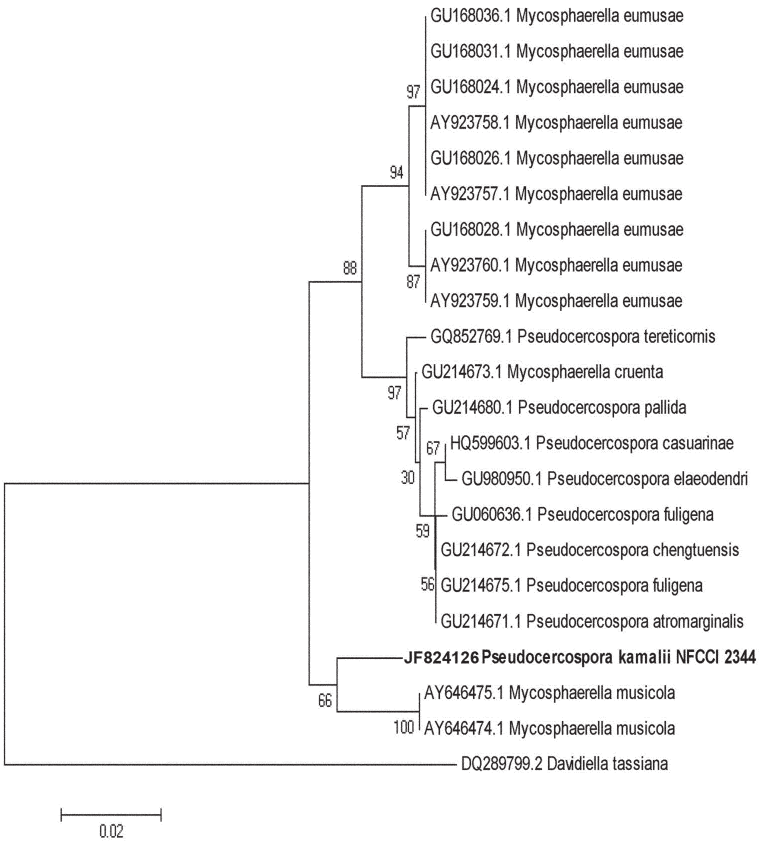


Fig. 2. Phylogenetic tree based on aligned ITS sequences of *Pseudocercospora* and its teleomorph, *Mycosphaerella* s.l. and inferred using the Neighbor-Joining method in MEGA. The optimal tree with the sum of branch length = 0.25106088 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option).

Taxonomy

The new cercosporoid species on *Terminalia chebula* is easily distinguishable from all *Pseudocercospora* species hitherto described on *Terminalia* by its synnematous conidiomata, and hence it is described here as new species.

Pseudocercospora kamalii Rajeshkumar, Rahul Sharma & S.K. Singh, **sp. nov.**

MycoBank MB 561201 Figs. 3–4

Synnemata 200–337.5 µm longa, ex stromatibus oriunda. Stromata bene evoluta, globosa, subglobosa vel irregularia, atrobrunnea. 50–72 µm diam., ex cellulis oblongis, cylindraceis composita. Conidiophora 130–263 × 5–6 µm, modice brunnea vel atrobrunnea, crassitunicata, pluriseptata. Conidia solitaria, sicca, in multitudine atro-brunnea, 25–57.5 × 5.5–8 µm, laevia, raro verruculosa, crassitunicata, 2–7 septis crassis, basi truncate.

Type: India, Mahabaleshwar, Western Ghats, Maharashtra, on leaves of *Terminalia chebula* Retz. (*Combretaceae*), January 2011, K.C. Rajeshkumar & Rahul Sharma (AMH 9425, **holotype**; ex-type culture NFCCI 2344.)

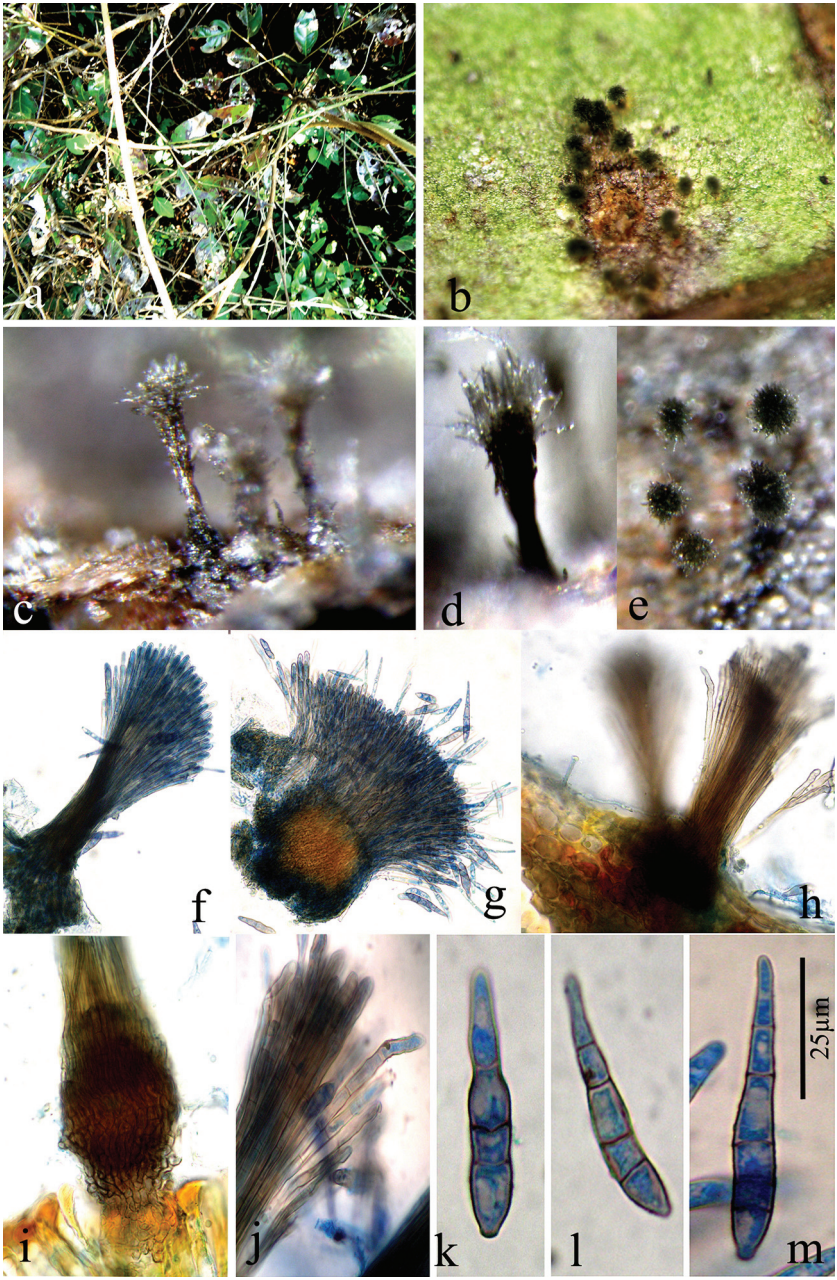
Etymology: *kamalii,* named in honour of Prof. Dr. Kamal, Emeritus Professor, Department of Botany, D.D.U. Gorkhpur University for his major contribution to the cercosporoid fungi of India.

Leaf spots necrotic, amphigenous, circular, angular or irregular, forming concentric dark and pale brown patterns on the spots, spreading eventually covering most of the leaf, but not vein limited, 1–3 cm diam. Conidiomata synnematous and caespituli either exclusively hypogenous or amphigenous, synnemata blackish brown 200–337.5 µm long, arising from well developed stroma, globose, subglobose or irregular, dark brown or blackish brown, 50–72 µm diam, formed of elongated cylindrical cells. Conidiophores medium to dark brown, thick and dark-walled, formed as a continuation of stromatic cells, 130–263 µm long and 5–6 µm wide, 8–10 to multiseptate. Conidiogenous cells integrated, terminal, cylindrical or sometime doliiform, geniculate, slightly wider and paler towards truncate apex, which at times can be obtusely rounded; conidial scars 1–2 in number, 2–3 µm diam, slightly thicker and dark, conidial loci not usually protuberant. Conidia solitary, holoblastic, dry, dark brown to blackish brown in mass, pale brown when immature, later turning to medium to dark brown, 25–57.5 × 5.5–8 µm, straight or slightly curved, mostly smooth, rarely minutely verruculose, thick-walled, thick septate, 2–7-septate, conidial wall and septations are thicker and darker in the basal part, and pale and thin towards apex, conidial base truncate, 2.0–2.2 µm, and apex obtuse or subacute 2.5–3.5 µm

Teleomorph: not observed.

Colonies on MEA (Fig. 5) very slow growing, 0.5 to 0.8 mm diam after 7 days, grayish white or white initially, later turning dark grayish black to blackish brown, velutinous, reverse blackish to dark blackish brown. Colonies become 2 to 2.3 mm after 30 days, forming a sulcate pattern, and sectored on MEA.

Fig. 3. *Pseudocercospora kamalii* (holotype). a. Habit. b. Symptoms on leaves and synnemata. c–d. Synnemata side view. e. Synnemata top view. f–h. Conidioma with stroma. i. Well developed stroma. j. Conidiophore tip and conidial scar. k–m. Conidia with thick wall and thick septations. Bars: k, l, m = 25 µm.


a
b
c
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e
f
g
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i
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k
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m
25µm

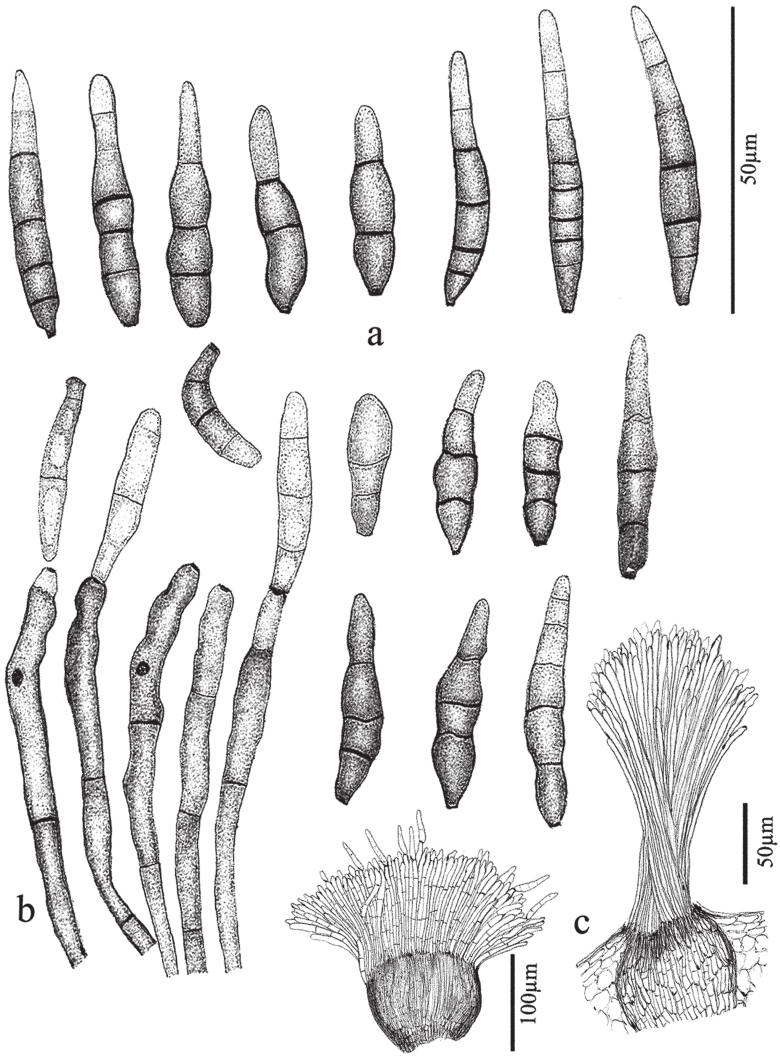

Fig. 4. *Pseudocercospora kamalii.* (holotype). a. Conidiophore and conidial development. b. Conidia. c. Synnemata

Culture on MEA not sporulating (lacking conidial development) but forming distinct synnemata-like bunches of conidiophores and dark celled stroma-like structures. The hyphae are medium to dark brown, highly verruculose and interwoven, forming a thick mat.

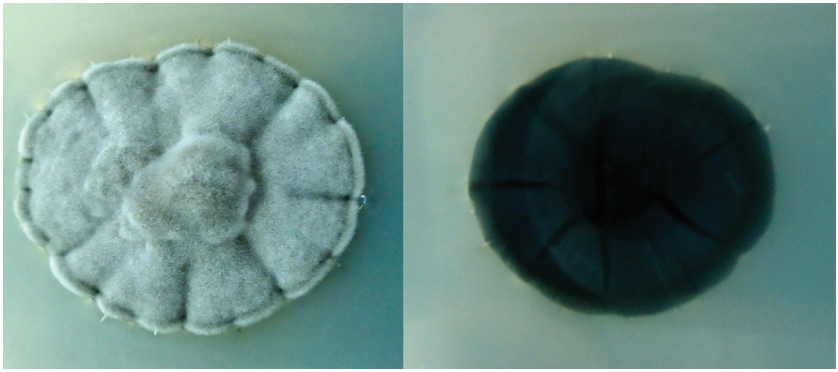

Fig. 5. *Pseudocercospora kamalii.* (holotype) Ex-type culture on MEA. a. Top view b. Reverse view.

Discussion

The new species, *Pseudocercospora kamalii,* is morphologically quite unusual and obscure within *Pseudocercospora,* as it exhibits many characters that match the genus *Prathigada.* Among these, the thick conidial septations and wall pattern are mostly identical with species of *Prathigada*. Only a few cercosporoid species with similar morphological characters (thick-walled, multi-septate, pigmented conidia containing slightly thickened and darkened scars) have been transferred to *Prathigada* (Braun 1996; Furlanetto & Dianese 1999; Sutton 1994), most being retained in *Pseudocercospora*. Thus far, 15 species are recorded under the genus *Prathigada.* Initial morphological studies also made it evident that *Pseudocercospora kamalii* is morphologically close to *Prathigada terminaliae* (Syd.) B. Sutton recorded on *Terminalia* spp. (Sutton 1994). However, *P. kamalii* has synnematous conidiomata, smaller conidia, and fewer conidial septa. Furthermore, the LSU and ITS sequence analyses accurately defined the species boundaries and placed *P. kamalii* into *Pseudocercospora.* Crous et al. (2009b) recently stated that due to the unavailability of cultures, no decision could yet be made on the phylogenetic placement of several less well-known genera such as *Prathigada* Subram. within the *Mycosphaerellaceae*. A recollection, epitypification and detailed phylogenetic analysis of the type species, *Prathigada cratevae* (Syd.) Subram. on *Crateva religiosa* G. Forst. from India, is needed to reveal the exact relation of the genus *Prathigada* with *Pseudocercospora* and their placement in *Mycosphaerellaceae*.

Sutton (1994) also reported six *Pseudocercospora* taxa on *Terminalia* spp. from India: *P. arjunae* B. Sutton (on *T. arjuna*), *P. brevis* B. Sutton (on *T. bellerica*), *P. catappae* (Henn.) Y.L. Guo & X.J. Liu (on *T. catappa*, *T. tomentosa*, *T. arjuna*), *P. chebulae* B. Sutton (on *T. chebula*), *P. combretacearum*

R.K. Verma & Kamal var. *combretacearum* (on *T. bellerica*, *Terminalia* sp.), and *P. combretacearum* var. *minima* B. Sutton (on *T. bellerica*); he also reported two other species: *P. neodeightonii* B. Sutton (on *T. albida* from Sierra Leone) and *P. zambiensis* (Deighton) B. Sutton (on *T. mollis* from Zambia). Kamal (2010: 347) listed *P. catappae, P. chebulae,* and *P. combretacearum* var. *combetacearum* from Indian *T. chebula.* These three species differ from *P. kamalii* in various morphological characters; most conspicuously, they have mononematous, fasciculate, or sporodochial conidiophores in contrast with the synnematous conidiophores of *P. kamalii*, which is the only synnematous *Pseudocercospora* species recorded on *Terminalia*.

In the present study, *P. kamalii* is proposed as a new species based on the LSU & ITS sequence analysis (Fig. 1, 2) and morphological characterization (Fig. 3, 4). Phylogenetically, *P. kamalii* is closely related to *Pseudocercospora musae* (66% bootstrap support) on *Musa* spp. (Arzanlou et al. 2008, Braun et al. 1999). With its distinct synnemata with a well-developed basal stroma and larger conidiophores and conidia, *P. kamalii* is morphologically distinct from *Pseudocercospora musae* and all other taxa with higher phylogenetic similarity.

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